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4. Title of the invention "SCHIZOPHRENIA ASSOCIATED GENE (II)"

5. Name of your agent *(if you have one)* CRUIKSHANK & FAIRWEATHER

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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CRUIKSHANK & FAIRWEATHER 4 APRIL 2002

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# SCHIZOPHRENIA ASSOCIATED GENE (II)

The present invention relates to the identification of a gene which has been disrupted in a patient diagnosed as suffering from comorbid schizophrenia and mild learning disability, or mental retardation (US terminology), as well as proteins encoded by the gene and antibodies thereto and to uses of such products as medicaments for treating schizophrenia and/or affective psychosis. The invention also relates to methods for diagnosing patients suffering or predisposed to schizophrenia and/or affective psychosis, as well as screens for developing novel treatment regimes for schizophrenia and/or affective psychosis.

Schizophrenia is a common and debilitating psychiatric disorder. Despite a wealth of information on the epidemiology, neuroanatomy and pharmacology of the illness, it is uncertain what molecular pathways are involved and how impairments in these affect brain development and neuronal function. Despite an estimated heritability of 60-80%, very little is known about the number or identity of genes involved in these psychoses. Although there has been recent progress in linkage and association studies, especially from genome-wide scans, these studies have yet to progress from the identification of susceptibility loci or candidate genes to the full characterisation of disease-causing genes (Berrettini, 2000).

The cloning of breakpoints in patients with chromosome abnormalities (translocations, inversions etc.) has proved instrumental in the identification of many disease genes (e.g. Duchenne Muscular Dystrophy, Retinoblastoma, Wilm's Tumour, Familial Polyposis Coli, Fragile-X Syndrome, Polycystic Kidney Disease, many leukaemias and, very recently, a candidate speech and language disorder gene (Lai et al, 2001)). Such studies assume that the chromosomal breakpoints give rise to the clinical symptoms by either directly disrupting gene sequences or perturbing gene expression. In the latter case, it is considered that the breakpoint can still affect expression of a gene at a distance of at least up to 1Mb (Kleinjan & van Heyningen, 1998). In the same way that gene-trap mutagenesis can be used to identify disrupted mouse genes (Brennan & Skarnes, 1999), the physical "flag" created by a cytogenetic breakpoint provides a geographical pointer for the disease locus.

It is amongst the objects of the present invention to provide a gene and/or protein postulated to be involved with the development and/or symptoms associated with schizophrenia and/or affective psychosis.

The present invention is based on the GRIK4 gene and observations of the present inventors of an involvement of this gene and/or protein with schizophrenia and/or affective psychosis.

The GRIK4 gene is also known as KA1 and EAA1, but will herein be referred to as GRIK4 for simplicity, but should not be construed as limiting.

As will be seen, the present invention is based on the molecular characterisation of a chromosomal rearrangement in a subject diagnosed as suffering from schizophrenia. A high-throughput Fluorescence *in situ* Hybridisation (FISH)-based approach has been adopted to map the chromosomal breakpoints in these patients. Consultation of the sequence data at the breakpoint locus not only allows efficient FISH probe selections to be made by the targeting of coding regions, but proof of gene disruption can be made entirely by relating the exact position of probes to the genomic structure of a candidate gene. This removes the necessity for laborious physical mapping of breakpoint loci, a process that requires quantities of genomic DNA that are not necessarily available from some psychiatric patients or their derived lymphoblastoid cell lines.

The subject was one of a series of around 100 patients with comorbid schizophrenia and mild learning disability (US terminology: "mental retardation") who were screened using routine G-band karyotyping. It has been repeatedly observed that schizophrenia occurs more frequently in individuals with mild learning disability than in the general population and recent work has revealed an increased heritability of this comorbid state.

As described herein the FISH results reveal that the subject has a disruption in a brain expressed gene; namely, *GRIK4* which is known to participate in molecular mechanisms responsible for modulating the strength of synaptic transmission.

In a first aspect the present invention provides use of a polynucleotide fragment comprising the *GRIK4* gene or a fragment, derivative or homologue thereof for the manufacture of a medicament for treating schizophrenia and/or affective disorder in a subject.

In another aspect the present invention provides use of a polypeptide fragment encoded by the *GRIK4* gene, or a fragment, derivative or homologue thereof for the manufacture of a medicament for treating schizophrenia and/or effective psychosis.

Schizophrenia and/or affective psychosis as used herein relates to schizophrenia, as well as other affective psychoses such as those listed in "The ICD-10 Classification of Metal and Behavioural Disorders" World Health Organization, Geneva 1992. Categories F20 to F29 inclusive includes Schizophrenia, schizotypal and delusional disorders. Categories F30 to F39 inclusive are Mood (affective) disorders that include bipolar affective disorder and depressive disorder. Mental Retardation is coded F70 to F79 inclusive. The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). American Psychiatric Association, Washington DC. 1994. Include all conditions coded 295.xx (Schizophrenia and

Other Psychotic Disorders) and 296.xx (Major Depressive Disorders and Bipolar Disorders). Mental Retardation is coded 315, 317, 318 and 319.

The *GRIK4* gene is located on chromosome 11, at cytogenetic position 11q22.3. The gene encodes a kainate receptor subunit and has been previously described by Kamboj et al, 1994. The cDNA nucleotide sequence and peptide sequence was disclosed by Kamboj et al, 1994 and submitted to the Genbank/EMBL database under accession NM_014619. The coding sequence of the gene is identified as being 2871 nucleotides in length, coding for a protein 957 amino acids. The nucleotide and protein sequences are shown in Figures 1 and 2 respectively. The present inventors have identified an alternative start site for the gene (see Figures 6 - 9) which would result in a shorter gene/protein of 933 amino acids as opposed to 956. The full nucleotide sequence and protein sequence of this alternatively encoded gene/protein is shown in Figures 7 and 8.

Thus, references herein to the *GRIK4* gene are understood to relate to the sequences identified in Figures 1 and 7 and references to the *GRIK4* protein sequence are understood to relate to the sequences identified in Figures 2 and 8.

In certain jurisdictions claims to methods of treatment are permissible and so the skilled reader will appreciate that the *GRIK4* gene, or fragment, derivative or homologue thereof; or *GRIK4* protein, or functionally active

fragment, derivative, or homologue thereof, may be administered to an individual as a method of treating an individual with schizophrenia and/or affective psychosis.

"Polynucleotide fragment" as used herein refers to a chain of nucleotides such as deoxyribose nucleic acid (DNA) and transcription products thereof, such as RNA. Naturally, the skilled addressee will appreciate the whole naturally occurring human genome is not included in the definition of polynucleotide fragment.

The polynucleotide fragment can be isolated in the sense that it is substantially free of biological material with which the whole genome is normally associated *in vivo*. The isolated polynucleotide fragment may be cloned to provide a recombinant molecule comprising the polynucleotide fragment. Thus, "polynucleotide fragment includes double and single stranded DNA, RNA and polynucleotide sequences derived therefrom, for example, subsequences of said fragment and which are of any desirable length. Where a nucleic acid is single stranded then both a given strand and a sequence or reverse complementary thereto is within the scope of the present invention.

In general, the term "expression product" or "gene product" refers to both transcription and translation products of said polynucleotide fragments. When the expression product is a "polypeptide" (i.e. a chain or sequence of amino acids displaying a biological activity substantially similar (eg. 98%, 95%, 90%, 80%, 75%

activity) to the biological activity of the protein), it does not refer to a specific length of the product as such. Thus, the skilled addressee will appreciate that "polypeptide" encompasses *inter alia* peptides, polypeptides and proteins. The polypeptide if required, can be modified *in vivo* and *in vitro*, for example by glycosylation, amidation, carboxylation, phosphorylation and/or post-translational cleavage.

The present invention further provides a recombinant or synthetic polypeptide for the manufacture of reagents for use as therapeutic agents in the treatment of schizophrenia and/or affective psychosis. In particular, the invention provides pharmaceutical compositions comprising the recombinant or synthetic polypeptide together with a pharmaceutically acceptable carrier therefor.

The present invention further provides an isolated polynucleotide fragment capable of specifically hybridising to a related polynucleotide sequence from another species. In this manner, the present invention provides probes and/or primers for use in *ex vivo* and/or *in situ* detection and expression studies. Typical detection studies include polymerase chain reaction (PCR) studies, hybridisation studies, or sequencing studies. In principle any specific polynucleotide sequence fragment from the identified sequences may be used in detection and/or expression studies. The skilled addressee understands that a specific fragment is a fragment of the sequence which is of

sufficient length, generally greater than 10, 12, 14, 16 or 20 nucleotides in length, to bind specifically to the sequence, under conditions of high stringency, as defined herein, and not bind to unrelated sequences, that is sequences from elsewhere in the genome of the organism other than an allelic form of the sequence or non-homologous sequences from other organisms.

"Capable of specifically hybridising" is taken to mean that said polynucleotide fragment preferably hybridises to a related or similar polynucleotide sequence in preference to unrelated or dissimilar polynucleotide sequences.

The invention includes polynucleotide sequence(s) which are capable of specifically hybridising to an polynucleotide fragment as described herein or to a part thereof without necessarily being completely complementary or reverse complementary to said related polynucleotide sequence or fragment thereof. For example, there may be at least 50%, or at least 75%, at least 90%, or at least 95% complementarity. Of course, in some cases the sequences may be exactly reverse complementary (100% reverse complementary) or nearly so (e.g. there may be less than 10, typically less than 5 mismatches). Thus, the present invention also provides anti-sense or complementary nucleotide sequence(s) which is/are capable of specifically hybridising to the disclosed polynucleotide sequence. If a specific polynucleotide is to be used as a primer in PCR and/or sequencing studies, the polynucleotide must be capable of hybridising to related nucleic acid and capable

of initiating chain extension from 3' end of the polynucleotide, but not able to correctly initiate chain extension from unrelated sequences.

If a polynucleotide sequence of the present invention is to be used in hybridisation studies to obtain or identify a related sequence from another organism the polynucleotide sequence should preferably remain hybridised to a sample polynucleotide under stringent conditions. If desired, either the test or sample polynucleotide may be immobilised. Generally the test polynucleotide sequence is at least 10, 14, 20 or at least 50 bases in length. It may be labelled by suitable techniques known in the art. Preferably the test polynucleotide sequence is at least 200 bases in length and may even be several kilobases in length. Thus, either a denatured sample or test sequence can be first bound to a support. Hybridization can be effected at a temperature of between 50 and 70°C in double strength SSC (2xNaCl 17.5g/l and sodium citrate (SC) at 8.8g/l) buffered saline containing 0.1% sodium dodecyl sulphate (SDS). This can be followed by rinsing of the support at the same temperature but with a buffer having a reduced SSC concentration. Depending upon the degree of stringency required, and thus the degree of similarity of the sequences, such reduced concentration buffers are typically single strength SSC containing 0.1%SDS, half strength SSC containing 0.1%SDS and one tenth strength SSC containing 0.1%SDS. Sequences having the greatest degree of similarity are those the hybridisation of which is least

affected by washing in buffers of reduced concentration. It is most preferred that the sample and inventive sequences are so similar that the hybridisation between them is substantially unaffected by washing or incubation in standard sodium citrate (0.1 x SSC) buffer containing 0.1%SDS.

Oligonucleotides may be designed to specifically hybridise to GRIK4 nucleic acid. They may be synthesised, by known techniques and used as primers in PCR or sequencing reactions or as probes in hybridisations designed to detect the presence of a mutated or normal GRIK4 gene in a sample. The oligonucleotides may be labelled by suitable labels known in the art, such as, radioactive labels, chemiluminescent labels or fluorescent labels and the like.

The term "oligonucleotide" is not meant to indicate any particular length of sequence and encompasses nucleotides of preferably at least 10b (e.g. 10b to 1kb) in length, more preferably 12b-500b in length and most preferably 15b to 100b.

The oligonucleotides may be designed with respect to any of the sequences described herein and may be manufactured according to known techniques. They may have substantial sequence identity (e.g. at least 50%, at least 75%, at least 90% or at least 95% sequence identity) with one of the strands shown therein or an RNA equivalent, or with a part of such a strand. Preferably such a part is at least 10, at least 30, at least 50 or at least 200 bases

long. It may be an open reading frame (ORF) or a part thereof.

Oligonucleotides which are generally greater than 30 bases in length should preferably remain hybridised to a sample polynucleotide under one or more of the stringent conditions mentioned above. Oligonucleotides which are generally less than 30 bases in length should also preferably remain hybridised to a sample polynucleotide but under different conditions of high stringency. Typically the melting temperature of an oligonucleotide less than 30 bases may be calculated according to the formula of; 2°C for every A or T, plus 4°C for every G or C, minus 5°C. Hybridization may take place at or around the calculated melting temperature for any particular oligonucleotide, in 6 x SSC and 1% SDS. Non specifically hybridised oligonucleotides may then be removed by stringent washing, for example in 3 x SSC and 0.1% SDS at the same temperature. Only substantially similar matched sequences remain hybridised i.e. said oligonucleotide and corresponding test nucleic acid.

When oligonucleotides of generally less than 30 bases in length are used in sequencing and/or PCR studies, the melting temperature may be calculated in the same manner as described above. The oligonucleotide may then be allowed to anneal or hybridise at a temperature around the oligonucleotides calculated melting temperature. In the case of PCR studies the annealing temperature should be around the lower of the calculated melting temperatures for

the two priming oligonucleotides. It is to be appreciated that the conditions and melting temperature calculations are provided by way of example only and are not intended to be limiting. It is possible through the experience of the experimenter to vary the conditions of hybridisation and thus anneal/hybridise oligonucleotides at temperatures above their calculated melting temperature. Indeed this can be desirable in preventing so-called non-specific hybridisation from occurring.

It is possible when conducting PCR studies to predict an expected size or sizes of PCR product(s) obtainable using an appropriate combination of two or more oligonucleotides, based on where they would hybridise to the sequences described herein. If, on conducting such a PCR on a sample of DNA, a fragment of the predicted size is obtained, then this is predictive that the DNA encodes a homologous sequence from a test organism.

Proteins for all the applications described herein can be produced by cloning the gene for example into plasmid vectors that allow high expression in a system of choice e.g. insect cell culture, yeast, animal cells, bacteria such as *Escherichia coli*. To enable effective purification of the protein, a vector may be used that incorporates an epitope tag (or other "sticky" extension such as His6) onto the protein on synthesis. A number of such vectors and purification systems are commercially available.

The polynucleotide fragment can be molecularly cloned into a prokaryotic or eukaryotic expression vector using standard techniques and administered to a host. The expression vector is taken up by cells and the polynucleotide fragment of interest expressed, producing protein.

It will be understood that for the particular polypeptides embraced herein, natural variations such as may occur due to polymorphisms, can exist between individuals or between members of the family. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. All such derivatives showing the recognised activity are included within the scope of the invention. For example, for the purpose of the present invention conservative replacements may be made between amino acids within the following groups:

(I) Alanine, serine, threonine;

(II) Glutamic acid and aspartic acid;

(III) Arginine and leucine;

(IV) Asparagine and glutamine;

(V) Isoleucine, leucine and valine;

(VI) Phenylalanine, tyrosine and tryptophan

Moreover, recombinant DNA technology may be used to prepare nucleic acid sequences encoding the various derivatives outlined above.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in a different codon which is still capable of coding for the same amino acid, e.g. the codon for amino acid glutamic acid is both GAT and GAA. Consequently, it is clear that for the expression of polypeptides from nucleotide sequences described herein or fragments thereof, use can be made of a derivative nucleic acid sequence with such an alternative codon composition different from the nucleic acid sequences shown in the Figures.

The polynucleotide fragments of the present invention are preferably linked to regulatory control sequences. Such control sequences may comprise promoters, operators, inducers, enhancers, silencers, ribosome binding sites, terminators etc. Suitable control sequences for a given host may be selected by those of ordinary skill in the art.

A polynucleotide fragment according to the present invention can be ligated to various expression controlling sequences, resulting in a so called recombinant nucleic acid molecule. Thus, the present invention also includes an expression vector containing an expressible nucleic acid molecule. The recombinant nucleic acid molecule can then be used for the transformation of a suitable host.

Specific vectors which can be used to clone nucleic acid sequences according to the invention are known in the art (e.g. Rodriguez, R.L. and Denhadt, D.T., Edit., Vectors: a survey of molecular cloning vectors and their uses, Butterworths, 1988, or Jones *et al.*,Vectors: Cloning

Applications: Essential Techniques (Essential techniques series), John Wiley & Son. 1998).

The methods to be used for the construction of a recombinant nucleic acid molecule according to the invention are known to those of ordinary skill in the art and are inter alia set forth in Sambrook, et al. (Molecular Cloning: a laboratory manual Cold Spring Harbour Laboratory, 1989).

The present invention also relates to a transformed cell containing the polynucleotide fragment in an expressible form. "Transformation", as used herein, refers to the introduction of a heterologous polynucleotide fragment into a host cell. The method used may be any known in the art, for example, direct uptake, transfection transduction or electroporation (Current Protocols in Molecular Biology, 1995. John Wiley and Sons Inc.). The heterologous polynucleotide fragment may be maintained through autonomous replication or alternatively, may be integrated into the host genome. The recombinant nucleic acid molecules preferably are provided with appropriate control sequences compatible with the designated host which can regulate the expression of the inserted polynucleotide fragment, e.g. tetracycline responsive promoter, thymidine kinase promoter, SV-40 promoter and the like.

Suitable hosts for the expression of recombinant nucleic acid molecules may be prokaryotic or eukaryotic in origin. Hosts suitable for the expression of recombinant nucleic acid molecules may be selected from bacteria,

yeast, insect cells and mammalian cells.

In another aspect the present invention also relates to a method of diagnosing schizophrenia and/or affective psychosis or susceptibility to schizophrenia and/or affective psychosis in an individual, wherein the method comprises determining if the *GRIK4* gene in the individual has been disrupted by a mutation or chromosomal rearrangement.

The methods which may be employed to elucidate such a mutation or chromosomal rearrangement are well known to those of skill in the art and could be detected for example using PCR or in hybridisation studies using suitable probes which could be designed to span an identified mutation site or chromosomal breakpoint in the *GRIK4* gene, such as the breakpoint identified by the present inventors and described herein.

Once a particular polymorphism or mutation has been identified it may be possible to determine a particular course of treatment. For example it is known that some forms of treatment work for some patients, but not all. This may in fact be due to mutations in the *GRIK4* gene or surrounding sequence, and it may therefore be possible to determine a treatment strategy using current therapies, based on a patient's genotype.

It will be appreciated that mutations in the gene sequence or controlling elements of a gene, eg. a promoter and/or enhance can have subtle effects such as affecting mRNA splicing/stability/activity and/or control of gene

expression levels, which can also be determined.

Also the relative levels of RNA can be determined using for example hybridisation or quantitative PCR as a means to determine if the *GRIK4* gene has been disrupted.

Moreover the presence and/or levels of the *GRIK4* gene products themselves can be assayed by immunological techniques such as radioimmunoassay, Western blotting and ELISA using specific antibodies raised against the gene products. The present invention also therefore relates to antibodies specific for a *GRIK4* gene product and uses thereof in diagnosis and/or therapy.

A further aspect of the present invention therefore provides antibodies specific to the polypeptides of the present invention or epitopes thereof. Production and purification of antibodies specific to an antigen is a matter of ordinary skill, and the methods to be used are clear to those skilled in the art. The term antibodies can include, but is not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanised or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope binding fragments of any of the above. Such antibodies may be used in modulating the expression or activity of the particular polypeptide, or in detecting said polypeptide *in vivo* or *in vitro*.

Using the sequences disclosed herein, it is possible to identify related sequences in other animals, such as mammals, with the intention of providing an animal model for psychiatric disorders associated with the improper functioning of the nucleotide sequences and proteins of the present invention. Once identified, the homologous sequences can be manipulated in several ways common to the skilled person in order to alter the functionality of the nucleotide sequences and proteins homologous to those of the present invention. For example, "knock-out" animals may be created, that is, the expression of the genes comprising the nucleotide sequences homologous to those of the present invention may be reduced or substantially eliminated in order to determine the effects of reducing or substantially eliminating the expression of such genes. Alternatively, animals may be created where the expression of the nucleotide sequences and proteins homologous to those of the present invention are upregulated, that is, the expression of the genes comprising the nucleotide sequences homologous to those of the present invention may be increased in order to determine the effects of increasing the expression of these genes. In addition to these manipulations, substitutions, deletions and additions may be made to the nucleotide sequences encoding the proteins homologous to those of the present invention in order to effect changes in the activity of the proteins to help elucidate the function of domains, amino acids, etc. in the proteins. Furthermore, the sequences of the present

invention may also be used to transform animals to the manner described above. The manipulations described above may also be used to create an animal model of schizophrenia and/or affective psychosis associated with the improper functioning of the nucleotide sequences and/or proteins of the present invention in order to evaluate potential agents which may be effective for combatting psychiotic disorders, such as schizophrenia and affective disorder psychosis.

Thus, the present invention also provides for screens for identifying agents suitable for preventing and/or treating schizophrenia and/or affective psychosis associated with disruption or alteration in the expression of the *GRIK4* gene and/or its gene products. Such screens may easily be adapted to be used for the high throughput screening of libraries of compounds such as synthetic, natural or combinatorial compound libraries.

Thus, the *GRIK4* gene products according to the present invention can be used for the *in vivo* or *in vitro* identification of novel ligands or analogs thereof. For this purpose binding studies can be performed with cells transformed with nucleotide fragments according to the invention or an expression vector comprising a polynucleotide fragment according to the invention, said cells expressing the *GRIK4* gene products according to the invention.

Alternatively also the *GRIK4* gene products according to the invention as well as ligand-binding domains thereof can be used in an assay for the identification of

functional ligands or analogs for the *GRIK4* gene products.

Methods to determine binding to expressed gene products as well as *in vitro* and *in vivo* assays to determine biological activity of gene products are well known. In general, expressed gene product is contacted with the compound to be tested and binding, stimulation or inhibition of a functional response is measured.

Thus, the present invention provides for a method for identifying ligands for *GRIK4* gene products, said method comprising the steps of:

a) introducing into a suitable host cell a polynucleotide fragment according to the invention;

b) culturing cells under conditions to allow expression of the polynucleotide fragment;

c) optionally isolating the expression product;

d) bringing the expression product (or the host cell from step b)) into contact with potential ligands which will possibly bind to the protein encoded by said polynucleotide fragment from step a);

e) establishing whether a ligand has bound to the expressed protein; and

f) optionally isolating and identifying the ligand.

As a preferred way of detecting the binding of the ligand to the expressed protein, also signal transduction capacity may be measured.

Compounds which activate or inhibit the function of *GRIK4* gene products may be employed in therapeutic treatments to activate or inhibit the polypeptides of the

present invention.

The present invention will now be further described by way of Example and with reference to the Figures which show:

**Figure 1** shows the published nucleotide sequence for GRIK4.

**Figure 2** shows the published amino acid sequence for GRIK4.

**Figure 3** Breakpoints identified in the subject. CEPH library YACs (Chumakov et al, 1992) spanning the breakpoints are listed. Also detailed are the BAC clones (and accession numbers) from the RPCI-11 BAC library (Osoegawa et al, 2001) that span or flank (indicated by dashes) the breakpoints. Breakpoints at 8q13 were not characterised in this study.

**Figure 4** Representation of complex chromosomal rearrangement in the subject. The pericentric chromosome 2 inversion is coupled with a translocation to chromosome 11. The chromosome 11 region between the 11q23.3 and 11q24.3 breakpoints is inserted on chromosome 8q13.

**Figure 5** Genomic arrangements of the GRIK4 gene disrupted in the subject. Two potential *GRIK4* transcripts with alternative start-sites are indicated. The 1a/1a' exons are derived from EST BE388730. The transcript containing the 1b exon corresponds to the published *GRIK4* sequence (acc. S67803). It is probable that the present inventors exon "4" corresponds to a number of undefined exons which can only be subdivided after release of genomic

sequence over this part of the gene. Hence, the actual number of *GRIK4* transcript exons will most likely exceed 14. BAC (grey boxes), cosmid (white boxes) and long-range PCR product (black line) derived FISH probes enabled the positioning of the breakpoint (arrows indicate the relative direction of the breakpoint deduced from the presence/absence of the signals on the two derived chromosomes). Probes from BAC RPCI-11 89P5 and cosmids LA11197-C5, LA1163-H6, LA11236-G3 and LA1192-C6 indicated that the breakpoint was located near exons 2 and 3. A FISH probe synthesized from a long-range PCR product corresponding to the intronic sequence between these two exons indicated that the breakpoint lies upstream of the intron between exons 2 and 3.

**Figure 6** 5' sequence of the *GRIK4* gene showing the two possible N-terminal peptides derived from alternate start sites. Exon combination 1a-1a'-2 is derived from an EST sequence (acc. BE388730). Exon combination 1b-2 is based on the published cDNA sequence (e.g. acc. S67803). The actual amino acid sequence may differ from the published amino acid sequence as there is a potential downstream methionine start (MVAC... instead of MPRV...) containing a more conserved Kozak sequence (Kozak, 1986). It can be seen that the breakpoint upstream of exon 2 will separate the majority of the coding sequence from the promoter resulting in a putative null allele. Exonic DNA sequence is shown in capitals, intronic or upstream sequence in lower case. Conserved splice junction sequences (EXON/GT-----AG/EXON)

are underlined. Single letter amino acid codes are shown beneath the appropriate DNA codons. A functional C/G:Leu/Val single nucleotide polymorphism (underlined) is found within exon 2.

**Figure 7** shows the complete alternative nucleic acid sequence as identified by the present inventors.

**Figure 8** shows the complete alternative amino acid sequence as identified by the present inventors.

## Materials and Methods

### Psychiatric evaluation

The subject (female) was approached and gave full, informed written consent for this study as one of a large cohort of people co-morbid for schizophrenia and mental retardation. Prior to investigation she was not known to have any abnormality of karyotype. She suffered from chronic schizophrenia and a mild degree of mental retardation (IQ between 65-70). The diagnosis of chronic schizophrenia was confirmed using SADS-L structured interview to generate DSM-IV and ICD-10 criteria, by a psychiatrist experienced in both general psychiatry and the psychiatry of mental retardation (WM). SADS can be reliably used in patients with mild mental retardation. Consensus diagnosis was reached on review by two psychiatrists (WM and DB). IQ scores were generated from WAIS-R and their stability shown by similar levels detected by psychological examination at different times throughout

her life. There were no dysmorphic features in the subject. However the subject did suffer from bilateral deafness since childhood - a consequence of surgical operations on the mastoids. There was no family history of mental illness or mental retardation that could be ascertained. Other members of the family declined to participate in the study.

**Lymphocyte extraction and metaphase chromosome preparation**

Lymphocytes were extracted from 7mls of patient blood (for storage and generation of EBV-transformed cell lines) using density gradient separation (Histopaque-1077, Sigma). In order to generate metaphase-arrested chromosomes for cytogenetic analysis, 0.8mls of patient blood were cultured for 71hrs in medium containing phytohaemagglutinin (Peripheral Blood Medium, Sigma). The short-term cultures were treated with colcemid for one hour followed by a conventional fixing procedure. Fixed chromosomes were dropped onto microscope slides and stored for 1 week prior to use in FISH experiments.

**Selection of YAC clones for FISH probe synthesis**

YAC clones were selected from the Whitehead/MIT map of the relevant chromosome in the cytogenetic intervals within which the breakpoints were adjudged to lie. YACs were obtained from the HGMP Resource Centre, Babraham Bioincubator, Babraham, Cambridge, UK (http://www.hgmp.mrc.ac.uk/). Clone DNA was prepared by

standard methods and PCR amplified using primers designed against consensus sequence elements within the archetypal *Alu* repeat (Breen et al, 1992). This "Alu-PCR" gives a representative spread of non-repetitive sequence over the full length of the YAC and generates a better FISH probe than native YAC DNA. Alu-PCR was performed using the Expand Long Template PCR kit (Roche). Cycling conditions:94°C - 45s, 55°C - 30s, 68°C - 8min: 35 cycles. 68°C - 10min final extension.

**Fluorescence *in situ* hybridisation (FISH) protocol**

Probe template DNA (pooled Alu-PCR products, BAC clone DNA, cosmid clone DNA or long-range PCR products) was labelled by nick translation and hybridised to patient metaphase spreads using standard FISH methods. Slides were counterstained with DAPI in Vectashield anti-fade solution (Vector laboratories). The Zeiss Axioskop fluorescence microscope with a chroma number 81000 multi-spectral filter set. Images were captured using Vysis SmartCapture extension running within IP Lab spectrum. FISH signals observed on derived chromosomes dictated the selection of further clones required to "walk" towards the breakpoint. Breakpoint-spanning FISH probes have signals on a normal chromosome and on both derived chromosomes.

**Resolution of breakpoint position**

BAC clones corresponding to positive YAC regions were arranged into contigs by consulting the Washington

University FPC (http://www.genome.wustl.edu/gsc/human/Mapping/index.shtml), UCSC GoldenPath Draft Human Genome Browser (http://genome.ucsc.edu/goldenPath/hgTracks.html) and Ensembl (http://www.ensembl.org/) databases. BAC clones were supplied by BACPAC Resources, Oakland, California, USA (http://www.chori.org/bacpac/). Clone selection was biased to gene-containing BACs. Once a breakpoint-spanning BAC was identified, the position of the breakpoint in relation to candidate gene exons was determined by FISH probes generated from chromosome-specific library cosmids (HGMP Resource centre) or precisely positioned, repeat element-free long-range PCR products (Expand long range PCR kit, Roche; see below for primer sequences). Cycling conditions:94°C - 45s, 52°C - 30s, 68°C - 11min: 35 cycles. 68°C - 15min final extension. Cosmids were isolated by probing the appropriate chromosome-specific library filters (HGMP-RC) with isotopically labelled exon-specific PCR products.

**PCR primers**

Long-range PCR for FISH probe templates:

Int2-3 GRIK4a; CAGGAGGTCCTGTGAAGCTC,

Int2-3 GRIK4b; ACAGGGAAAGAAGCAAAGCA.

GRIK4 exon region-specific PCR: screening of chromosome 11 cosmid libraries:

Ex1a/a' a; AAAGCTAAGCGCAGGTGTGT,

Ex1a/a' b; TTTCTGGGAGGCAACCATAG,

Ex1b a; GCAGAGTTATGTCATGCCCA,

Ex1b b; CCTGTGCAGCACTCTGATGT,

Ex2/3 a; TTGAACCCAAGAGAACAGGG,

Ex2/3 b; TCCCCTTCTCCTTCCAGTTT

Cycling conditions:94°C - 2min initial denaturation. 94°C - 1min, 52°C - 1min, 72°C - 75s: 33 cycles. 72°C - 15min final extension.

**Example 1: Molecular characterisation of chromosomal disruption and identification of disrupted gene**

An initial G-banded karyotype of this patient indicated that the chromosome abnormality was complex (46, XX,ins(8;11)(q13;q23.3q24.2)inv(2)(p12q32.1) t(2;11)(q21.3;q24.2)der(2)(2qter->2q32.1::2p12->2q21.3::11q24.2->11qter) der(11)(11pter->11q23.3::2q21.3->2q32.1::2p12->2pter)der(8)(8pter->8q13::11q23.3->11q24.2::8q13->8qter)), involving a pericentric inversion of chromosome 2 coupled with rearrangements involving chromosomes 2, 8 and 11 (Fig.4). Figure 3 details the YAC and BAC FISH probes crossing or bracketing breakpoints on 2 and 11. Sequence in the locality of the breakpoints was assessed for gene content.

The 11q23.3 breakpoint is located at a locus containing a kainate-type ionotropic glutamate receptor (*GRIK4*, acc. S67803 & NM_014619 (11), previous nomenclature *KA1/EAA1*). Cosmid FISH directed at the individual exons

and an intron-specific long-range PCR product FISH (Fig.6) positioned the breakpoint within the *GRIK4* gene sequence; most likely immediately upstream of exon 2 (our nomenclature, Fig.6). This was confirmed using a long-range PCR product FISH probe corresponding to the intron between exons 2 and 3 (Fig.6). We also identified a GenBank EST (acc. BE388730, IMAGE clone ID:3613199) generating an alternative start-site resulting in an alternative cognate N-terminal peptide sequence (Figures 7 and 9). The position of a breakpoint anywhere between exons1a/a'/1b and exon 3 would truncate all putative transcript forms such that no receptor function could be encoded on the derived chromosome 11. Hence, the patient had only one intact *GRIK4* allele.

**Discussion**

The present inventors identified a subject with comorbin schizophrenia with mild learning disability in whom chromosome translocation events have disrupted brain-expressed gene that are also functional disease candidates. Without wishing to be bound by theory it is hypothesised that the disruption of the GRIK4 gene by a chromosomal breakpoint (and the resulting reduced gene dosage) is the principal underlying cause of psychiatric disease in this patient.

The gene disrupted in this patient is both expressed in the brain and participates in key physiological processes in the CNS. Notably, the gene may be involved in

the alteration of the strength of synaptic/neural transmission, a phenomenon known as long-term potentiation (LTP). LTP is postulated to underlie cognitive functions such as learning and memory. Moreover, cognitive testing has previously established that these functions are frequently affected in patients with schizophrenia.

*GRIK4*

Three classes of ionotropic glutamate receptors have been identified on the basis of their pharmacological profiles and sequence homologies; NMDA receptors, AMPA receptors and Kainate receptors. Functional Kainate receptors *in vivo* may be heteromeric, consisting of combinations of the low kainate agonist affinity (GLUR5, GLUR6 and GLUR7) and high-affinity subunits (GRIK4 and GRIK5) (Chittajallu et al, 1999; Lerma et al, 2001 and Werner et al, 1991). The subject with comorbid schizophrenia and mild learning disability possesses a complex chromosomal rearrangement. Of all the breakpoints studied in this patient only the *GRIK4* gene is directly disrupted. This might be expected to modify kainate receptor channel properties by altering subunit stoichiometry.

The glutamate receptors are key initiators of synaptic LTP (Miller and Mayford, 1999). NMDA receptors are the principal mediators of LTP but recently presynaptic kainate receptor-dependent plasticity changes have been observed at mossy fibre synapses in the hippocampus (Contractor et al,

2001 and Lauri et al, 2001). Interestingly, an involvement of the glutamate neurotransmitter system in the pathophysiology of schizophrenia has been postulated. The "Glutamate Hypothesis" attempts to explain the psychotic symptoms that arise following administration of ionotropic glutamate receptor antagonists such as phencyclidine (PCP; "Angel Dust") and ketamine (Goff and Nine, 1997). Several studies also point to changes, predominantly decreases, in glutamate receptor subunit expression (including kainate receptors) in the brains of schizophrenic patients (Ibrahim et al, and Meador-Woodruff, 2001). Similarly, Mohn et al, 1999 report that mutant mice with reduced NMDAR1 (another glutamate receptor) expression levels display schizophrenia-like behaviours.

As well as aberrant neurotransmission function in the adult, it has been suggested that neurodevelopmental deficits may contribute to schizophrenia. Neuroanatomical studies indicate statistically significant reduced volumes of brain regions, primarily the hippocampus, in schizophrenic and comorbid patients (Sanderson et al 1999 and Pearlson, 1999). *GRIK4* is expressed in the amygdala, hippocampal formation (CA3 pyramidal and dentate granule cells) and entorhinal cortex. Glutamate receptors might mediate brain development through the activity-dependent refinement of neuronal connections.

The present subject was clinically diagnosed as having schizophrenia coupled with mild learning disability. It may be the case that causative gene mutations in comorbid

patients lead to a severe phenotype or have more profound downstream effects than gene mutations in patients with schizophrenia alone (i.e. the comorbid state represents the severest form of schizophrenia (Doody et al, 1998)). A second possibility is that the gene mutation gives rise to the learning disability component of the illness through an independent effect on brain development. The manner in which the mutated genotype gives rise to the observed phenotype (via functional or developmental mechanisms) is a key issue in molecular neurobiology, particularly in the characterisation of mouse "knockout" mutants (Mayford et al, 1995).

A large number of publications detail family and population-based linkage studies carried out to identify psychiatric illness susceptibility loci. The results have not been conclusive perhaps indicating the presence of confounding factors such as population stratification, incomplete penetrance, genetic heterogeneity and uncertain mode of inheritance. Nevertheless, *GRIK4* lies at the edge of a schizophrenia linkage region described in a recent publication (Gurling et al, 2001). The most centromeric marker exhibiting linkage to schizophrenia in this paper, D11S925, is located within an intron at the 3' end of *GRIK4*.

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# Figure 1

Published GRIK4 nucleic acid sequence (accession NM_014619).

```
   1 atgccccgcg tctcggcgcc tttggtgctg cttcctgcgt ggctcgtgat ggtcgcctgc
  61 agcccgcact ccttgaggat cgctgctatc ttggacgacc ccatggagtg cagcagaggg
 121 gagcggctct ccatcaccct ggccaagaac cgcatcaacc gcgctcctga gaggctgggc
 181 aaggccaagg tcgaagtgga catctttgag cttctcagag acagcgagta cgagactgca
 241 gaaaccatgt gtcagatcct ccccaagggg gtggtcgctg tcctcggacc atcgtccagc
 301 ccagcctcca gctccatcat cagcaacatc tgtggagaga aggaggtccc tcacttcaaa
 361 gtggccccag aggagttcgt caagttccag ttccagagat tcacaaccct gaacctccac
 421 cccagcaaca ctgacatcag cgtggctgta gctgggatcc tgaacttctt caactgcacc
 481 accgcctgcc tcatctgtgc caaagcagaa tgcctttaa acctagagaa gctgctccgg
 541 caattcctta tctccaagga cacgctgtcc gtccgcatgc tggatgacac ccgggacccc
 601 accccgctcc tcaaggagat ccgggacgac aagaccgcca ccatcatcat ccacgccaac
 661 gcctccatgt cccacaccat cctcctgaag gcagccgaac ttgggatggt gtcagcctat
 721 tacacataca tcttcactaa tctggagttc tcactccaga gaacggacag ccttgtggat
 781 gatcgtgtca acatcctggg attttccatt ttcaaccaat cccatgcttt cttccaagag
 841 tttgcccaga gcctcaacca gtcctggcag gagaactgtg accatgtgcc cttcactggg
 901 cctgcgctct cctcggccct gctgtttgat gctgtctatg ctgtggtgac tgcggtgcag
 961 gaactgaacc ggagccaaga gatcggcgtg aagcccttgt cctgcggctc ggcccagatc
1021 tggcagcacg gcaccagcct catgaactac ctgcgcatgg tagaattgga aggtcttacc
1081 ggccacattg aattcaacag caaaggccag aggtccaact acgctttgaa aatcttacag
1141 ttcacaagga atggttttcg gcagatcggc cagtggcacg tggcagaggg cctcagcatg
1201 gacagccacc tctatgcctc caacatctcg gacactctct tcaacaccac cctggtcgtc
1261 accaccatcc tggaaaaccc atatttaatg ctgaagggga accaccagga gatggaaggc
1321 aatgaccgct acgagggctt ctgtgtggac atgctcaagg agctggcaga gatcctccga
1381 ttcaactaca agatccgcct ggttggggat ggcgtgtacg gcgttcccga ggccaacggc
1441 acctggacgg gaatggtcgg ggagctgatc gctaggaaag cagatctggc tgtggcaggc
1501 ctcaccatta cagctgaacg ggagaaggtg attgatttct ctaagccatt catgactctg
1561 ggaattagca ttctttaccg cattcatatg ggacgcaaac ccggctattt ctccttcctg
1621 gacccatttt ctccgggcgt ctggctcttc atgcttctag cctatctggc cgtcagctgt
1681 gtcctcttcc tggtggctcg gttgacgccc tacgagtggt acagcccaca cccatgtgcc
1741 cagggccggt gcaacctcct ggtgaaccag tactccctgg gcaacagcct ctggtttccg
1801 gtcgggggct tcatgcagca gggctccacc atcgcccctc gcgccttatc cacccgctgt
1861 gtcagtggcg tctggtgggc attcacgctg atcatcatct catcctacac ggccaacctg
1921 gcagccttcc tgaccgtgca gcgcatggat gtgcccattg agtcagtgga tgacctggct
1981 gaccagaccg ccattgaata tggcacaatt cacggaggct ccagcatgac cttcttccaa
2041 aattcccgct accagaccta ccaacgcatg tggaattaca tgtattccaa gcagcccagc
2101 gtgttcgtga agagcacaga ggagggaatc gccagggtgt tgaattccaa ctacgccttc
2161 ctcctggaat ccaccatgaa cgagtactat cggcagcgaa actgcaacct cactcagatt
2221 gggggcctgc tggacaccaa gggctatggg attggcatgc cagtcggctc ggttttccgg
2281 gacgagtttg atctggccat tctccagctg caggagaaca accgcctgga gatcctgaag
2341 cgcaaatggt gggaaggagg gaagtgcccc aaggaggaag atcacagagc taaaggcctg
2401 ggaatggaga atattggtgg aatctttgtg gttcttattt gtggcttaat cgtggccatt
2461 tttatggcta tgttggagtt tttatggact ctcagacact cagaagcaac tgaggtgtcc
2521 gtctgccagg agatggtgac cgagctgcgc agcattatcc tgtgtcagga cagtatccac
2581 ccccgccggc ggcgcgccgc agtcccgccg ccccggcccc ccatccccga ggagcgccga
2641 ccgcggggca cggcgacgct cagcaacggg aagctgtgcg gggcagggga gcccgaccag
2701 ctcgcgcaga gactggcgca ggaggccgcc ctggtggccc gcggctgcac gcacatccgc
2761 gtctgccccg agtgccgccg cttccagggc ctgcgggcac ggccgtcgcc cgcccgcagc
2821 gaggagagcc tggagtggga gaaaaccacc aacagcagcg agcccgagta g
```

## Figure 2

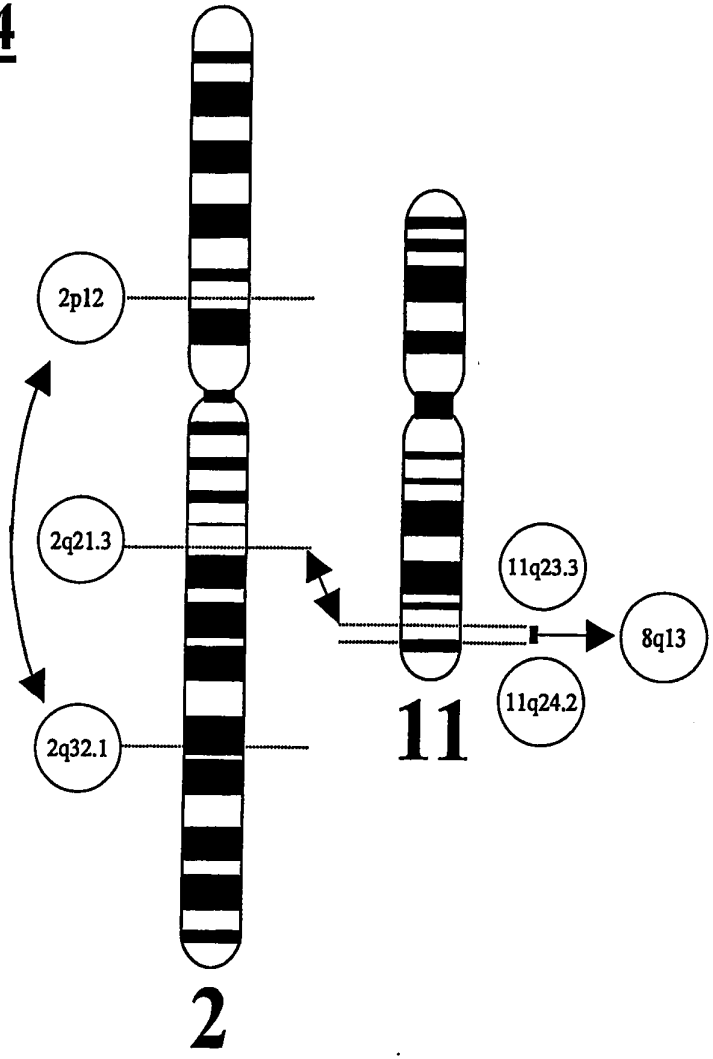
Published GRIK4 protein sequence (accession NP_055434).

MPRVSAPLVLLPAWLVMVACSPHSLRIAAILDDPMECSRGERLSITLAKNRINRAPE
RLGKAKVEVDIFELLRDSEYETAETMCQILPKGVVAVLGPSSSPASSSIISNICGEK
EVPHFKVAPEEFVKFQFQRFTTLNLHPSNTDISVAVAGILNFFNCTTACLICAKAEC
LLNLEKLLRQFLISKDTLSVRMLDDTRDPTPLLKEIRDDKTATIIIHANASMSHTIL
LKAAELGMVSAYYTYIFTNLEFSLQRTDSLVDDRVNILGFSIFNQSHAFFQEFAQSL
NQSWQENCDHVPFTGPALSSALLFDAVYAVVTAVQELNRSQEIGVKPLSCGSAQIWQ
HGTSLMNYLRMVELEGLTGHIEFNSKGQRSNYALKILQFTRNGFRQIGQWHVAEGLS
MDSHLYASNISDTLFNTTLVVTTILENPYLMLKGNHQEMEGNDRYEGFCVDMLKELA
EILRFNYKIRLVGDGVYGVPEANGTWTGMVGELIARKADLAVAGLTITAEREKVIDF
SKPFMTLGISILYRIHMGRKPGYFSFLDPFSPGVWLFMLLAYLAVSCVLFLVARLTP
YEWYSPHPCAQGRCNLLVNQYSLGNSLWFPVGGFMQQGSTIAPRALSTRCVSGVWWA
FTLIIISSYTANLAAFLTVQRMDVPIESVDDLADQTAIEYGTIHGGSSMTFFQNSRY
QTYQRMWNYMYSKQPSVFVKSTEEGIARVLNSNYAFLLESTMNEYYRQRNCNLTQIG
GLLDTKGYGIGMPVGSVFRDEFDLAILQLQENNRLEILKRKWWEGGKCPKEEDHRAK
GLGMENIGGIFVVLICGLIVAIFMAMLEFLWTLRHSEATEVSVCQEMVTELRSIILC
QDSIHPRRRRAAVPPPRPPIPEERRPRGTATLSNGKLCGAGEPDQLAQRLAQEAALV
ARGCTHIRVCPECRRFQGLRARPSPARSEESLEWEKTTNSSEPE

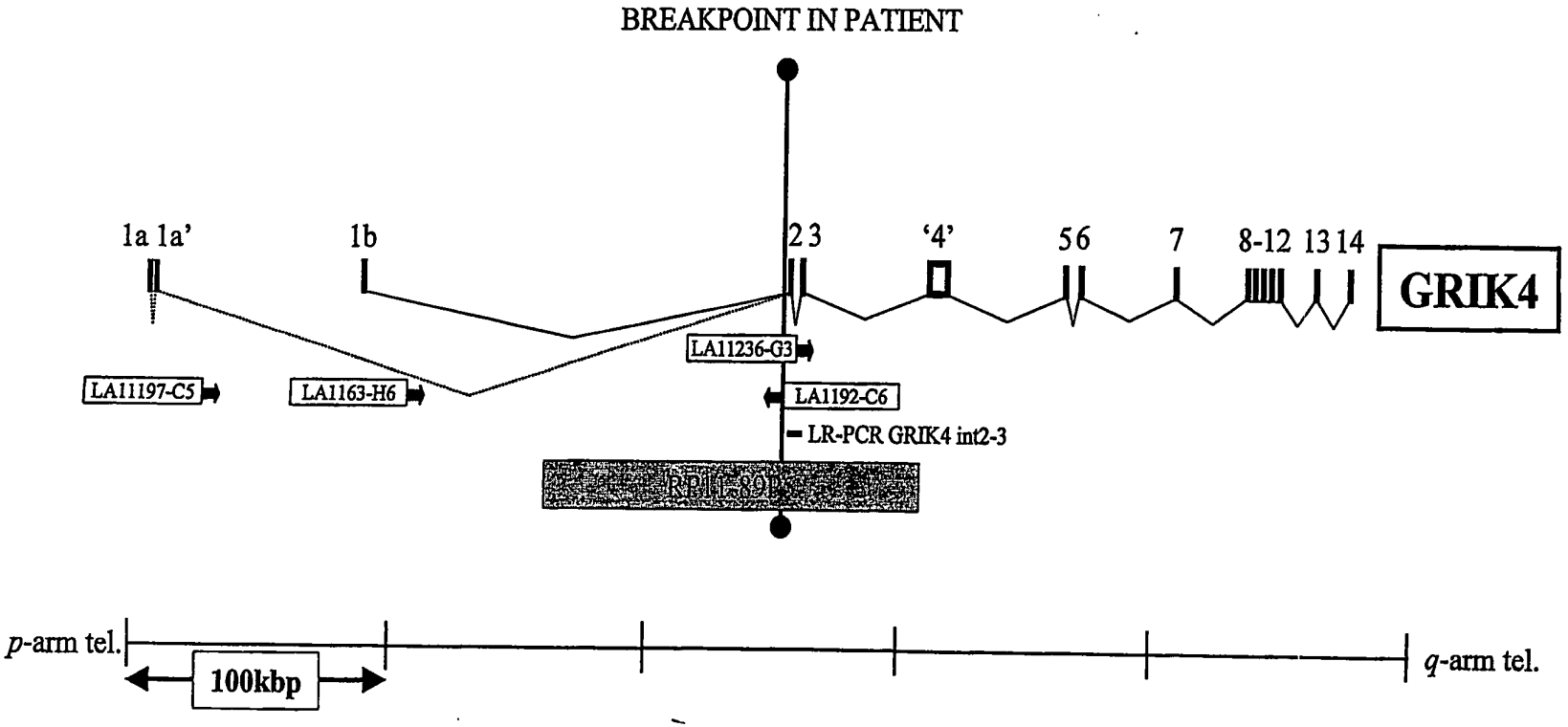
## Figure 3

| Patient | Cytogenetic Position | Description | Breakpoint YAC Clones | Breakpoint BAC Clones (Acc. No.) |
|---|---|---|---|---|
| 1 | 2p12 | Inversion breakpoint | 915 f 7 | - |
| 1 | 2q32.1 | Inversion breakpoint | 941 h 12 | RP11-358M9 (AC020595) |
| 1 | 2q21.3 | Translocation breakpoint | 766 c 12 | RP11-250H22 (AC011996) |
| 1 | 11q23.3 | Upper insertion breakpoint | 936 d 9 | RP11-89P5 (AC009641) |
| 1 | 11q24.2 | Translocation/Insertion breakpoint | 749 d 2 | RP11-687M24 (AP001007) |

**Figure 4**



# Figure 5


BREAKPOINT IN PATIENT
1a 1a'
1b
2 3
'4'
5 6
7
8-12 13 14
GRIK4
LA11236-G3
LA11197-C5
LA1163-H6
LA1192-C6
LR-PCR GRIK4 int2-3
RP11-50I
p-arm tel.
100kbp
q-arm tel.

# Figure 6

Exons from the two alternative splice forms of the GRIK4 mRNA. One form consists of exons 1a-1a'-2-etc and the other of exons 1b-2-etc. Amino acid codes are shown immediately beneath the corresponding nucleic acid codons. Lower case bases indicate intronic sequences. Underlined sequences indicate conserved splice sequences.

## Exon 1a

GCGTGGTAGCATGTGCCTGTAATCCCAGTGCTTTGGGACACCGAGGCAGGAGGATCACTCGAGCCCAGGAGTGCGAGGCTGCAgtg
agttatgatcatac

## Exon 1a'

agatttgtcttctctgccagGTGACGCTAGACTTCAGGAAGACCCCCCATTTCTGCTCCACTCCTGGGCTTGGAGAAGAGTACAGC
TGCTCTTGACTGGTGGGACCTTTTGCTGGCTAGGGGTGATGGGAGAAGCAAGAGAGGGATCCACACACCTGCGCTTAGCTTTCTAT
GACCTGGGCGGATGGAGGCCAAAGgtaaggtgggatgaga
M E A K A

## Exon 1b

CCATGAGGATTCATAGAAGATGCCCCGCGTCTCGGCGCCTTTGGTGCTGCTTCCTGCGTGGCTCGTGATGGTCGCCTGCAGCCCGC
M P R V S A P L V L L P A W L V M V A C S P H

ACTCCTTGAGGATCGgtaagtgtggcccagct
S L R I A

## Exon 2

gaaaccccccccagCTGCTATCTTGGACGACCCCATGGAGTGCAGCAGAGGGGAGCGGCTCTCCATCACCCTGGCCAAGAACCGCA
A I L D D P M E C S R G E R L S I T L A K N R I

TCAACCGCGCTCCTGAGAGGCTGGGCAAGGCCAAGGTCGAAGTGGACATCTTTGAGCTTCTCAGAGACAGCGAGTACGAGACTGCA
N R A P E R L G K A K V E V D I F E L L R D S E Y E T A

GAAACCAgtacgtagactggg
E T M

## Figure 7

Complete alternative nucleic acid sequence. Exons 1a-1a'-2-etc.

```
   1 gcgtggtagc atgtgcctgt aatcccagtg ctttgggaca ccgaggcagg aggatcactc
  61 gagcccagga gtgcgaggct gcagtgacgc tagacttcag gaagaccccc catttctgct
 121 ccactcctgg gcttggagaa gagtacagct gctcttgact ggtgggacct tttgctggct
 181 aggggtgatg ggagaagcaa gagagggatc cacacacctg cgcttagctt tctatgacct
 241 gggcggatgg aggccaaagc tgctatcttg gacgacccca tggagtgcag cagaggggag
 301 cggctctcca tcaccctggc caagaaccgc atcaaccgcg ctcctgagag gctgggcaag
 361 gccaaggtcg aagtggacat ctttgagctt ctcagagaca gcgagtacga gactgcagaa
 421 accatgtgtc agatcctccc caaggggtg gtcgctgtcc tcggaccatc gtccagccca
 481 gcctccagct ccatcatcag caacatctgt ggagagaagg aggtccctca cttcaaagtg
 541 gccccagagg agttcgtcaa gttccagttc cagagattca caaccctgaa cctccacccc
 601 agcaacactg acatcagcgt ggctgtagct gggatcctga acttcttcaa ctgcaccacc
 661 gcctgcctca tctgtgccaa agcagaatgc cttttaaacc tagagaagct gctccggcaa
 721 ttccttatct ccaaggacac gctgtccgtc cgcatgctgg atgacacccg ggaccccacc
 781 ccgctcctca aggagatccg ggacgacaag accgccacca tcatcatcca cgccaacgcc
 841 tccatgtccc acaccatcct cctgaaggca gccgaacttg ggatggtgtc agcctattac
 901 acatacatct tcactaatct ggagttctca ctccagagaa cggacagcct tgtggatgat
 961 cgtgtcaaca tcctgggatt ttccattttc aaccaatccc atgctttctt ccaagagttt
1021 gcccagagcc tcaaccagtc ctggcaggag aactgtgacc atgtgccctt cactgggcct
1081 gcgctctcct cggccctgct gtttgatgct gtctatgctg tggtgactgc ggtgcaggaa
1141 ctgaaccgga gccaagagat cggcgtgaag cccttgtcct gcggctcggc ccagatctgg
1201 cagcacggca ccagcctcat gaactacctg cgcatggtag aattggaagg tcttaccggc
1261 cacattgaat tcaacagcaa aggccagagg tccaactacg ctttgaaaat cttacagttc
1321 acaaggaatg gttttcggca gatcggccag tggcacgtgg cagagggcct cagcatggac
1381 agccacctct atgcctccaa catctcggac actctcttca acaccaccct ggtcgtcacc
1441 accatcctgg aaaacccata tttaatgctg aaggggaacc accaggagat ggaaggcaat
1501 gaccgctacg agggcttctg tgtggacatg ctcaaggagc tggcagagat cctccgattc
1561 aactacaaga tccgcctggt tggggatggc gtgtacggcg ttcccgaggc caacggcacc
1621 tggacgggaa tggtcgggga gctgatcgct aggaaagcag atctggctgt ggcaggcctc
1681 accattacag ctgaacggga gaaggtgatt gatttctcta agccattcat gactctggga
1741 attagcattc tttaccgcat tcatatggga cgcaaacccg gctatttctc cttcctggac
1801 ccatttttctc cgggcgtctg gctcttcatg cttctagcct atctggccgt cagctgtgtc
1861 ctcttcctgg tggctcggtt gacgccctac gagtggtaca gcccacaccc atgtgcccag
1921 ggccggtgca acctcctggt gaaccagtac tccctgggca acagcctctg gtttccggtc
1981 gggggttca tgcagcaggg ctccaccatc gcccctcgcg ccttatccac ccgctgtgtc
2041 agtggcgtct ggtgggcatt cacgctgatc atcatctcat cctacacggc caacctggca
2101 gccttcctga ccgtgcagcg catggatgtg cccattgagt cagtggatga cctggctgac
2161 cagaccgcca ttgaatatgg cacaattcac ggaggctcca gcatgacctt cttccaaaat
2221 tcccgctacc agacctacca acgcatgtgg aattacatgt attccaagca gcccagcgtg
2281 ttcgtgaaga gcacagagga gggaatcgcc agggtgttga attccaacta cgccttcctc
2341 ctggaatcca ccatgaacga gtactatcgg cagcgaaact gcaacctcac tcagattggg
2401 ggcctgctgg acaccaaggg ctatgggatt ggcatgccag tcggctcggt tttccgggac
2461 gagtttgatc tggccattct ccagctgcag gagaacaacc gcctggagat cctgaagcgc
2521 aaatggtggg aaggagggaa gtgccccaag gaggaagatc acagagctaa aggcctggga
2581 atggagaata ttggtggaat ctttgtggtt cttatttgtg gcttaatcgt ggccattttt
2641 atggctatgt tggagttttt atggactctc agacactcag aagcaactga ggtgtccgtc
2701 tgccaggaga tggtgaccga gctgcgcagc attatcctgt gtcaggacag tatccacccc
2761 cgccggcggc gcgccgcagt cccgccgccc cggcccccca tccccgagga gcgccgaccg
2821 cggggcacgg cgacgctcag caacgggaag ctgtgcgggg caggggagcc cgaccagctc
2881 gcgcagagac tggcgcagga ggccgccctg gtggcccgcg gctgcacgca catccgcgtc
2941 tgccccgagt gccgccgctt ccagggcctg cgggcacggc cgtcgcccgc ccgcagcgag
3001 gagagcctgg agtgggagaa aaccaccaac agcagcgagc ccgagtag
```

## Figure 8

Complete alternative protein sequence

MEAKAAILDDPMECSRGERLSITLAKNRINRAPERLGKAKVEVDIFELLRDSEYETA
ETMCQILPKGVVAVLGPSSSPASSSIISNICGEKEVPHFKVAPEEFVKFQFQRFTTL
NLHPSNTDISVAVAGILNFFNCTTACLICAKAECLLNLEKLLRQFLISKDTLSVRML
DDTRDPTPLLKEIRDDKTATIIIHANASMSHTILLKAAELGMVSAYYTYIFTNLEFS
LQRTDSLVDDRVNILGFSIFNQSHAFFQEFAQSLNQSWQENCDHVPFTGPALSSALL
FDAVYAVVTAVQELNRSQEIGVKPLSCGSAQIWQHGTSLMNYLRMVELEGLTGHIEF
NSKGQRSNYALKILQFTRNGFRQIGQWHVAEGLSMDSHLYASNISDTLFNTTLVVTT
ILENPYLMLKGNHQEMEGNDRYEGFCVDMLKELAEILRFNYKIRLVGDGVYGVPEAN
GTWTGMVGELIARKADLAVAGLTITAEREKVIDFSKPFMTLGISILYRIHMGRKPGY
FSFLDPFSPGVWLFMLLAYLAVSCVLFLVARLTPYEWYSPHPCAQGRCNLLVNQYSL
GNSLWFPVGGFMQQGSTIAPRALSTRCVSGVWWAFTLIIISSYTANLAAFLTVQRMD
VPIESVDDLADQTAIEYGTIHGGSSMTFFQNSRYQTYQRMWNYMYSKQPSVFVKSTE
EGIARVLNSNYAFLLESTMNEYYRQRNCNLTQIGGLLDTKGYGIGMPVGSVFRDEFD
LAILQLQENNRLEILKRKWWEGGKCPKEEDHRAKGLGMENIGGIFVVLICGLIVAIF
MAMLEFLWTLRHSEATEVSVCQEMVTELRSIILCQDSIHPRRRRAAVPPPRPPIPEE
RRPRGTATLSNGKLCGAGEPDQLAQRLAQEAALVARGCTHIRVCPECRRFQGLRARP
SPARSEESLEWEKTTNSSEPE